Application of large-scale sequencing to marker discovery in plants

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Advances in DNA sequencing provide tools for efficient large-scale discovery of markers for use in plants. Discovery options include large-scale amplicon sequencing, transcriptome sequencing, gene-enriched genome sequencing and whole genome sequencing. Examples of each of these approaches and their potential to generate molecular markers for specific applications have been described. Sequencing the whole genome of parents identifies all the polymorphisms available for analysis in their progeny. Sequencing PCR amplicons of sets of candidate genes from DNA bulks can be used to define the available variation in these genes that might be exploited in a population or germplasm collection. Sequencing of the transcriptomes of genotypes varying for the trait of interest may identify genes with patterns of expression that could explain the phenotypic variation. Sequencing genomic DNA enriched for genes by hybridization with probes for all or some of the known genes simplifies sequencing and analysis of differences in gene sequences between large numbers of genotypes and genes especially when working with complex genomes. Examples of application of the above-mentioned techniques have been described.

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1. Introduction

Molecular genetic markers have found wide application in plants (Henry 1997). Molecular markers are used increasingly in plant breeding to guide selection and plant identification and play an important role in determining plant identity and or purity in forensic and industrial situations. Protection of intellectual property rights such as plant variety rights is strongly supported by the use of molecular markers, while research on population genetics, conservation genetics and evolutionary genetics of plant population also makes extensive use of molecular genetic markers (Henry 2005).

DNA-based molecular markers have largely replaced earlier biochemical markers as techniques for DNA analysis in plants have improved (Henry 2001, 2008). Methods for both the discovery and analysis of molecular markers have been improved (Henry and Edwards 2009) with advances in high-throughput analysis of genetic polymorphisms focusing most recently on single nucleotide polymorphisms (SNP) (Kharabian-Masouleh *et al.* 2009; Varshney *et al.* 2010). For some time the application of high-throughput SNP

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analysis technology was limited by the availability of plant genome DNA sequence information, and screening populations for DNA marker discovery employed a range of techniques to deal with the lack of direct sequence information (Cross *et al.* 2008). Recently dramatic advances in second- and third-generation DNA sequencing technology have removed this barrier (Thudi *et al.* 2012).

2. Advances in DNA sequencing technologies

2.1 *Sequencing strategies*

Plant genome sequencing has progressed rapidly since the first genome (*Arabidopsis thaliana*) was completed in 2000 (Arabidopsis Genome Initiative 2000). The 389 Mb rice genome was completed in 2004 (IRGSP International Rice Genome Sequencing Project 2005). These projects used the 'traditional' Sanger sequencing approach. A well-proven method for large genome sequencing is known as BAC-by-BAC (bacterial artificial chromosome) sequencing and involves the production of an overlapping tiling path of large genomic fragments (around 120,000 bases) maintained within BACs. An alternative cheaper, high-throughput approach is the whole genome shotgun (WGS) method, where the entire genome is fragmented into many smaller pieces that are individually sequenced, and computational algorithms are applied to assemble the complete genome sequence. Second-generation sequencing (SGS) methods allow huge increases in data output at much lower costs; however, the read quality is more variable. This variability is mainly overcome by increased depth of coverage, which is especially important during SNP marker discovery (Deschamps and Campbell 2010, Varshney *et al.* 2010).

The assembly of large plant genome sequences is very problematic with short-read technologies due to sequence repeats. Various experimental designs that can facilitate sequence assembly include longer reads, paired-end reads, mate-pair libraries (large insert libraries), RNA-Seq data, reduced representation libraries, optical mapping and genetic mapping (Birney 2011). Paired-end or mate-pair protocols help to overcome some of these limitations of short reads by providing information about relative location and orientation of a pair of reads. In paired-end sequencing, the actual ends of rather short DNA molecules (<1 kb) are determined, while mate-pair sequencing requires the preparation of special libraries. In these protocols, the ends of longer, size-selected molecules (e.g. 8, 12 or 20 kb) are connected with an internal adapter sequence in a circularization reaction (figure 1) (Kircher and Kelso 2010).

Sample preparations often involve methods to select targeted regions in multiple individuals or samples instead of sequencing one sample to excessive depth in order to reduce costs and take advantage of the large output capacities in a single sequencing process. These methods also called 'genome partitioning', 'enrichment' or 'genomic capture' are being utilized. For example, megabase-size candidate genomic regions, identified through linkage studies, can be sequenced to find the exact genes and mutations underlying the trait. This is facilitated by techniques such as hybridization to preselected probes, mRNA extraction or attachment of barcode (sometimes called 'index') sequences to the library molecules. Samples are separated later (computationally) based on their individual barcode sequence. Commercial kits presently contain from 12 to 384 unique indexes (Edwards and Henry 2011). The complexity of polyploid genome analysis has been reduced through chromosome sorting preparations (Imelfort *et al.* 2009a).

2.2 *Sequencing Technologies*

For decades, DNA sequencing relied on the Sanger di-deoxy method with gradual improvements in the technology. Beginning in the mid 2000s a series of new techniques were developed and continuously improved. These techniques have allowed SGS to generate sequence data in very large volumes much more rapidly and cost-effectively (Varshney *et al.* 2010). The major technologies are detailed in table 1 (figure 2). SGS technologies have delivered large volumes of data, but the lowest cost options have been very short sequence reads. Third-generation technologies based upon single molecular analysis are currently being developed commercially. These technologies promise a number of different advances, lower cost per analysis, longer reads and direct RNA sequencing and direct analysis of methylation. A range of different applications for SGS have been developed and applied to marker discovery in plants (table 2).

SGS methods follow a similar pattern for library preparation that can be summarized in three major steps: (1) random shearing of DNA, either via nebulization or sonication; (2) ligation of universal adapters at both ends of the sheared DNA fragments and (3) immobilization and amplification of the adapter-flanked fragments to generate clustered amplicons to serve as templates for the sequencing reactions (Shendure and Ji 2008). These platforms parallelize from thousands to millions of sequencing reactions. Through alternating cycles of base incorporation and image capture, short DNA sequences ranging in size from 25 to 500 bases are generated (table 2) (Deschamps and Campbell 2010).

The dominant SGS sequencing platforms are the Roche Genome Sequencer FLX System, ABI SOLiD and the Illumina platforms. The 454 Genome Sequencer was initially introduced in 2005 by 454 Life Sciences and subsequently acquired by Roche Diagnostics and renamed the Roche Genome Sequencer FLX System. This platform utilizes pyrosequencing technology parallelized on separate beads

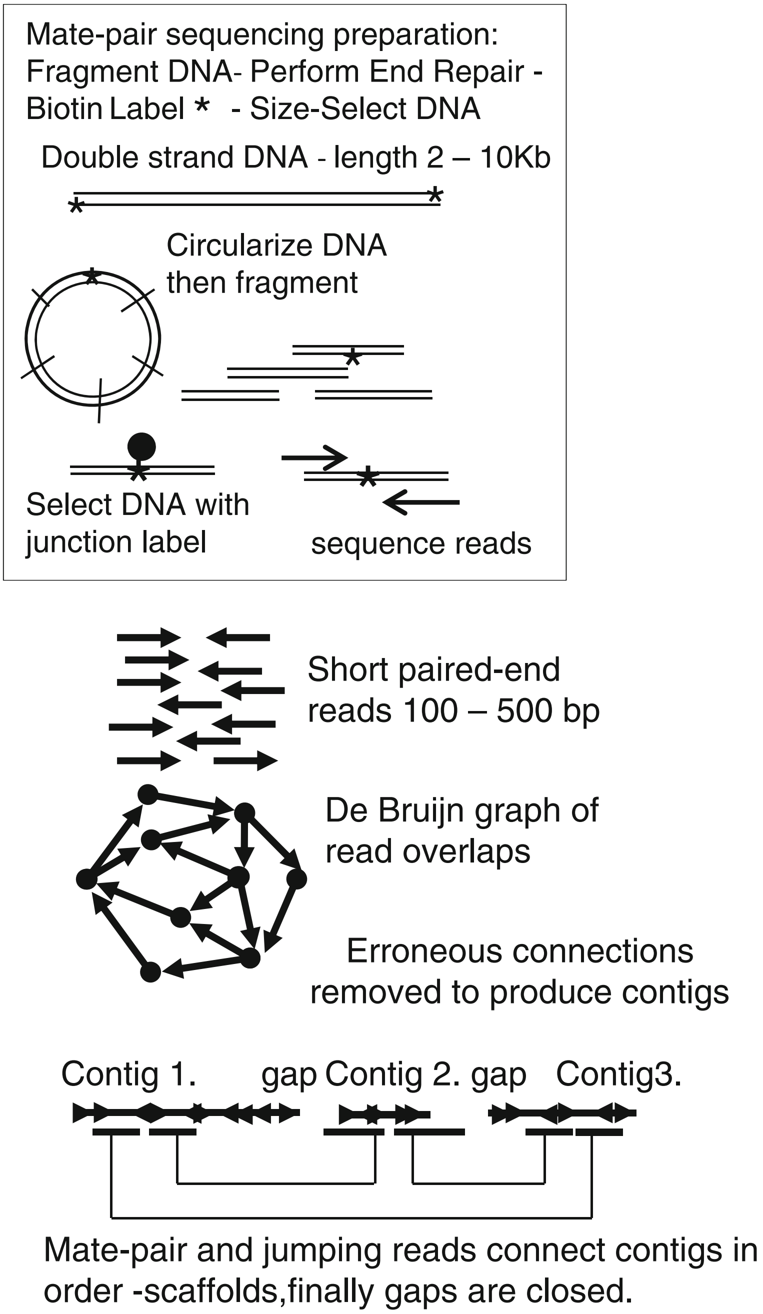

Mate-pair sequencing preparation:
Fragment DNA- Perform End Repair -
Biotin Label * - Size-Select DNA
Double strand DNA - length 2 – 10Kb
Circularize DNA
then fragment
Select DNA with
junction label
sequence reads
Short paired-end
reads 100 – 500 bp
De Bruijn graph of
read overlaps
Erroneous connections
removed to produce contigs
Contig 1.
gap
Contig 2.
gap
Contig3.
Mate-pair and jumping reads connect contigs in
order -scaffolds,finally gaps are closed.

Figure 1. Sequencing library preparations and *de novo* assembly of whole genome sequence requires a range of insert lengths using second-generation short read technologies (Glenn 2011).

in a picotiter plate. In pyrosequencing, one pyrophosphate per nucleotide is released and converted to ATP by an ATP sulphurylase. The ATP drives the light reaction of luciferases present (Kircher and Kelso 2010). The 454 sequencing at present produces the longest reads but the total sequence output per run is low (Brautigam and Gowik 2010; Magi *et al.* 2010). Illumina (former Solexa) sequencing introduced in early 2007 is the most widely used SGS technology. This method uses a solid-phase bridge PCR, and a ‘sequencing by synthesis’ approach, with fluorescent dye-labelled reversible terminator nucleotides allowing each cycle of the sequencing reaction to occur simultaneously in the presence of the four nucleotides that are distinguished by four fluorescent dyes. The ABI SOLiD (Sequencing by

Table 1. Advances in DNA sequencing technology as described by Edwards and Henry (2011)

Year of introduction	Platform	Maximum read length (2011 version)	Sequence Volume (approximate total per run)	Advances
1977	Sanger	1000 bp	100 Kbp	
Second-generation technologies				
2005	454 Life Sciences/Roche Dignostics	500 bp	500 Mbp	
2005	ABI SOLiD (Life Technologies)	50 bp	30 Gbp	
2007	Illumina Genome Analyser (Solexa)	150 bp	300 Gbp	
Third-generation technologies				
2010	Helicos (Helicos Biosciences)			Direct RNA sequencing
2010	Ion Torrent (Life Technologies)			Low-cost per analysis
2010	SMRT (Pacific Biosystems)			Long reads

Oligo Ligation and Detection) sequencer was introduced in 2005 by Agencourt Personal Genomics. This method employs sequential ligation producing short reads (up to 50 bases). The system features a two-base encoding mechanism that interrogates each base twice providing a form of built-in error detection (Brautigam and Gowik 2010).

The current SGS methods all have a requirement for numerous and slow wash–scan cycles. As each base is added, the population of molecules loses synchronicity (called dephasing), and this causes an increase in noise and sequencing errors as the read extends, effectively limiting the read length. In common with Sanger sequencing, the error rate also increases with sequence length due to a reduction in enzyme efficiency or loss of enzymes. In addition, to obtain sufficient light signal intensity for detection, the sequencing-by-synthesis reaction step requires PCR amplifications, which can introduce errors in the template sequence as well as amplification bias. Finally, the generation of huge amounts of sequence data has created challenges for storage and bioinformatics operations such as sequence annotation tasks (figure 3) (Imelfort *et al.* 2009a; Schadt *et al.* 2010).

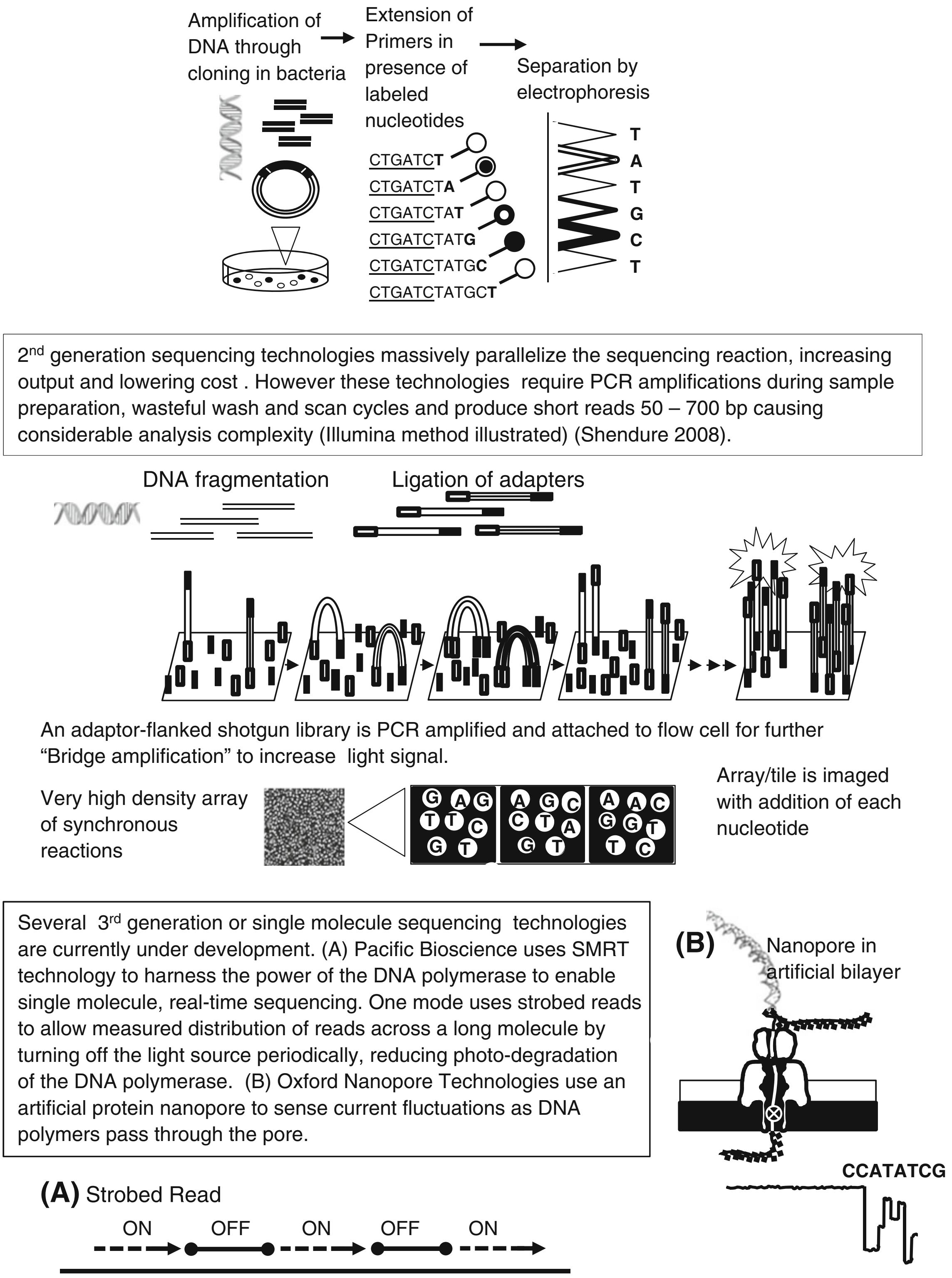
A recent survey of public and private sequencing laboratories in the US and Europe estimated an increase in utilization of SGS technologies from ~37% of to 56% within 2 years. Interest for third-generation platforms is expected to replace ~47% of all SGS activity in the next 3 years (Peterson *et al.* 2010). Third-generation DNA-sequencing technologies (table 1) are mostly distinguished by direct inspection of single molecules with methods that do not require the repetitive wash and scan steps during DNA synthesis, synchronization of multiple reactions or problems associated with PCR amplifications or phasing (Thudi *et al.* 2012). Several technologies are currently under development utilizing diverse approaches such as captured polymerases, nanopores, electronic detection and fluorescence resonance energy transfer (FRET) detection and transmission electron microscopy (TEM). Read lengths, time to results and cost are expected to greatly improve (Edwards and Henry 2011; Schadt *et al.* 2010).

3. Applications to plants

3.1 *Amplicon sequencing*

In plants, polymerase chain reaction (PCR) allows sequences of interest to be amplified and isolated from the genome, transcriptome or even organelles. Even with the limited capacity of first-generation (Sanger) sequencing, amplicons generated by PCR provided the basis for large scale analysis of allelic variations (SNPs and InDels) in genes, gene families and specific sequence regions of interest for population studies and marker development.

Advances in SGS have resulted in the continued application of amplicon sequencing in plant genetics (Bundock *et al.* 2009; Kulheim *et al.* 2009; Kharabian-Masouleh *et al.* 2011; Malory *et al.* 2011; Sexton *et al.* 2011). Because of the massive capacity of SGS, amplicons are now sequenced at coverage levels significantly higher than Sanger sequencing (deep sequencing). Deep sequencing utilizes the high sequencing capacity of SGS and ensures that base calling is internally validated, effectively allowing SNPs to be defined from sequencing errors (Bundock *et al.* 2009; Cronn *et al.* 2008). PCR amplification is used to isolate the sequence(s) of interest using individuals or pooled of DNA from the population of interest as a template. Amplicons from multiple genes or sequence regions can be combined (multiplexed or pooled) and sequenced together, so long as the genes are of sufficient difference to allow sequences to be assembled later by alignment of the sequence reads generated with the reference DNA sequences. Early application SGS to two different polyploid sugarcane genotypes identified 1632 and 1013 SNPs for the Q165 (242 amplicons) and


Amplification of DNA through cloning in bacteria
Extension of Primers in presence of labeled nucleotides
Separation by electrophoresis
CTGATCT
CTGATCTA
CTGATCTAT
CTGATCTATG
CTGATCTATGC
CTGATCTATGCT
T
A
T
G
C
T

2nd generation sequencing technologies massively parallelize the sequencing reaction, increasing output and lowering cost . However these technologies require PCR amplifications during sample preparation, wasteful wash and scan cycles and produce short reads 50 – 700 bp causing considerable analysis complexity (Illumina method illustrated) (Shendure 2008).

An adaptor-flanked shotgun library is PCR amplified and attached to flow cell for further “Bridge amplification” to increase light signal.

Very high density array of synchronous reactions

Array/tile is imaged with addition of each nucleotide

Several 3rd generation or single molecule sequencing technologies are currently under development. (A) Pacific Bioscience uses SMRT technology to harness the power of the DNA polymerase to enable single molecule, real-time sequencing. One mode uses strobed reads to allow measured distribution of reads across a long molecule by turning off the light source periodically, reducing photo-degradation of the DNA polymerase. (B) Oxford Nanopore Technologies use an artificial protein nanopore to sense current fluctuations as DNA polymers pass through the pore.

Figure 2. Early whole genome assembly used overlapping BACs (bacterial artificial chromosomes) with original Sanger sequencing technology (Kircher 2010).

Table 2. Applications of NGS to molecular discovery

Technique	Strategy for polymorphism discovery
Amplicon sequencing	Amplification of targeted genes or genome regions from different individuals or populations
Transcriptome sequencing	Sequencing of the transcriptome of different genotypes or tissues
Gene enriched genome sequencing	Sequencing of gene-rich genomic sequences from different genotypes
Whole genome sequencing	Re-sequencing of whole genomes of different genotypes
Organelle sequencing	Sequencing of organelle genomes from differ samples
Bisulfite sequencing	Sequencing of methylation pattern from different genotypes, tissues or conditions
Reduced representation	Sequencing of genomic regions across samples selected via restriction enzyme treatment

IJ76–514 (247 amplicons) varieties, respectively (Bundock *et al.* 2009). Cronn *et al.* (2008) used multiplex PCRs to amplify 35 amplicons representing ~120 kb of chloroplast sequence in individual genotypes representing seven Pinus and one Picea species. Following amplification genotypes were barcoded with 3 bp indexing tags and pooled prior to SGS. This experiment provided the basis for phylogenetic studies between many Pinus species (Parks *et al.* 2009; Whittall *et al.* 2010).

SGS can be effectively integrated with long-range PCR (LR-PCR) of pooled DNA samples. This maximizes the efficiency of sequencing and reduces the cost of amplification, oligonucleotides and labour (Out *et al.* 2009). Utilizing a LR-PCR approach, targeted deep sequencing can be employed to analyse regions surrounding candidate genes involved in various aspects of physiology, stress tolerance, quality, disease, etc. (Varley and Mitra 2008). This technology has the power to detect new mutations and discover rare alleles as a fragment of 10 kb may be read 100,000 times or more (Druley *et al.* 2009; Out *et al.* 2009; Thomas *et al.* 2006).

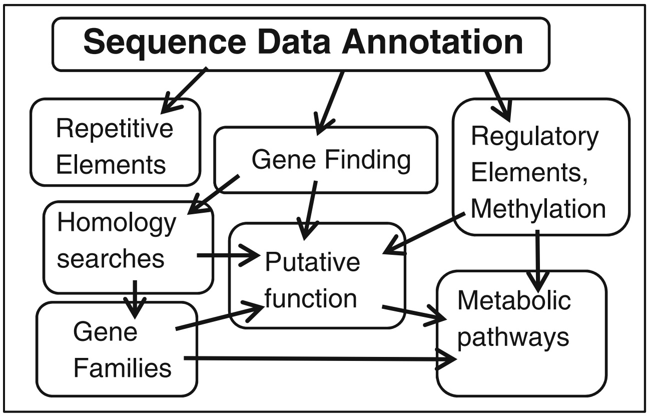


Figure 3. High-throughput assays using molecular markers facilitate plant identification and forensics and can guide early selection of a trait in a breeding program. Statistical analyses relate markers to physical characteristics. Innumerable marker-trait associations can be developed though marker effects can be inconsistent.

Pooling populations of individuals prior to sequencing, either at the tissue or DNA level, is another way to utilize the high sequencing capacity of SGS. By ensuring equal concentrations of DNA are pooled, accurate allele frequencies can be estimated for pooled populations (Out *et al.* 2009; Futschik and Schlotterer 2010; Sexton *et al.* 2011).Therefore, sequence polymorphisms can be identified between sequence reads bases of relative frequency (Kulheim *et al.* 2009; Kharabian-Masouleh *et al.* 2011; Malory *et al.* 2011; Sexton *et al.* 2011). In pooling experiments, genotype (individual) identities are confounded, and so it is important that pool size is not so high that target allele frequencies and expected error frequencies do not converge. For example, if 50 diploid genotypes are pooled, a rare allele in one haplotype is expected to occur at a frequency of 1%; if the pooling depth increases further, there is a risk that errors (e.g. Taq, sequencing, etc.) could be mistakenly confounded with SNP calls. Because of this issue it is not uncommon for researchers to only confirm SNPs at conservative allele frequencies of 5 to 10% (Novaes *et al.* 2008; Kulheim *et al.* 2009).

For several plant species trans-specific primers have been used to amplify orthologous genes in distantly related species not previously characterized. In conjunction with pooling and SGS-based amplicon sequencing, large-scale SNP discovery in candidate genes has been achieved for populations in species previously uncharacterized (Kulheim *et al.* 2009; Malory *et al.* 2011; Sexton *et al.* 2011). Using this approach with multiple species in parallel, researchers working with species in the diverse *Eucalyptus* genus have utilized comparative genomics for the discovery of shared (trans-specific) SNPs which may be of biological and commercial significance (Kulheim *et al.* 2009; Sexton *et al.* 2011). Subsequent association studies have validated SNPs identified with this method and also revealed several significant correlations with specific phenotypes (Kulheim *et al.* 2011; Sexton *et al.* 2012).

A similar pooling and amplicaon-based SGS approach was used to characterize genetic variation in starch biosynthesis genes, where the DNA of 233 rice genotypes was pooled and LR-PCR of 17 genes amplified 116,403 bp of genomic sequence. Subsequent SGS generated 60.9 million reads corresponding to the coverage between 12,000- and 38,000-fold for each of the genes, within the sequence reads 501 SNP and 113 InDels were identified (Kharabian-Masouleh *et al.* 2011). These SNPs have been related to starch properties explaining much of the variation in starch properties in cultivated rice (Kharabian-Masouleh *et al.* 2011 unpublished). Analysis of some of these genes in wild rice relatives is helping define the changes that have been introduced by human selection for desirable grain qualities.

A total of 399 SNPs for the targeted loci had already been reported at OryzaSNP@MSU database (*http://oryzasnp.plantbiology.msu.edu/*) domain for 20 rice cultivars. As expected, the total number of detected polymorphisms by Illumina sequencing was significantly higher than that reported in the rice public domain (OryzaSNP). Furthermore, discovery of InDels was another outcome of this approach as there were no InDels reported for these genes (Kharabian-Masouleh *et al.* 2011). A similar but more limited analysis of starch biosynthesis genes in sorghum germplasm using more laborious and expensive conventional sequencing has been used to explain variation in starch properties in sorghum (Hill *et al.* 2011).

3.2 *Transcriptome sequencing*

Transcriptome sequencing can not only define the sequences of all transcripts *de novo*, revealing previously undiscovered genes and splice variants, but can also indicate the relative abundance of the transcripts. This contrasts with traditional microarray analysis, which requires all genes of interest be known and represented on the microarray and does not return sequence/genotype data for a particular individual. Comparison of the transcriptome sequences of different tissues can be used follow changes in gene expression through development, in different tissues or in different genotypes to understand genetic differences that are due to differential gene expression (Gillies *et al.* 2012).

Genetic variation within commercial crop varieties is not usually well characterized or quantified. It follows then that the effect of intra-varietal genetic variation on crop performance under stress is also poorly understood, which may put production at risk from changing climate and rapidly evolving pests and diseases. Transcriptome sequencing allows genome-wide analysis of large, complex plant genomes and the potential to identify biologically significant SNPs. The genetic variation between and within barley varieties was defined by deep sequencing and assembled into unigenes the transcriptomes of two barley varieties Baudin and Gairdner (Pattemore *et al.* unpublished). A large number of SNP were identified, with more than 200,000 SNP between reads for variety Baudin and reference EST sequences, and more than 300,000 SNP between Baudin reads and *de novo* assembled reads from the variety Gairdner. Putatively significant SNPs (SNP allele frequency > 0.1) represented 9.65% and 14.64% of Baudin and Gairdner genetic variation. Background genetic diversity (SNP allele frequency ≤ 0.1) accounted for 90.23% and 85.52% of genetic variation in Baudin and Gairdner, respectively. The SNP dataset was further refined to produce a set of very high-quality SNPs for varietal genotyping. Although SNP variation within varieties has not been widely examined in other species, analyses of SNP between varieties have been undertaken to facilitate varietal distinction in many plant species like wheat (Trebbi *et al.* 2011), rice (Gopala Krishnan *et al.* 2012), maize (Barbazuk *et al.* 2007), chickpea (Hiremath *et al.* 2011), pigeonpea (Dubey *et al.* 2011), soybean (Wu *et al.* 2010) and oilseed rape (Trick *et al.* 2009) (see figure 4 for examples of SNP discovery strategies). Genome-wide marker sets, such as these developed from transcriptome sequencing, may become a valuable and complementary addition to the plant breeding toolbox uniquely aiding both selection and management of diversity within varieties.

3.3 *Whole genome sequencing*

Recent major improvements in whole genome shotgun sequencing has drastically reduced the cost of whole genome sequencing, providing low cost coverage of any genome, generating a sequence resource for molecular marker and gene discovery, comparative genomics and genome assembly. Draft genome sequences have been assembled based on second-generation sequencing technology in several crop species such as castor (Chan *et al.* 2010), pigeonpea (Dubey *et al.* 2011), cocoa (Argout *et al.* 2010). Assembly of reference genome sequences for complex plant genomes remains difficult (Souza *et al.* 2011). Coupled with the developments in bioinformatics to assemble short reads, next-generation sequencing technologies enables the discovery of millions of DNA polymorphisms such as SNPs and Indels by comparing the whole genome sequences of several individuals within a species. SNPs and InDels are becoming the preferred markers in molecular breeding due to multiple advantages such as high frequency, stability, high-throughput capability and cost-effectiveness over other DNA markers (Henry and Edwards 2009). An approach based on annotation has been developed for discovery of genome-wide SNPs without a reference genome in *Aegilops tauschii*, a diploid progenitor of the hexaploid wheat (Yan *et al.* 2011). More recently, high-quality reference of autotetraploid potato has been achieved by whole genome sequencing of homozygous doubled monoploid derived from conventional tissue culture and has been compared with its heterozygous diploid breeding line for SNP discovery and evolution of potato (TPGSC 2011).

Whole genome sequencing accelerates the rate of gene and molecular marker discovery. For instance, large-scale SNP markers have been developed in a wide array of crops

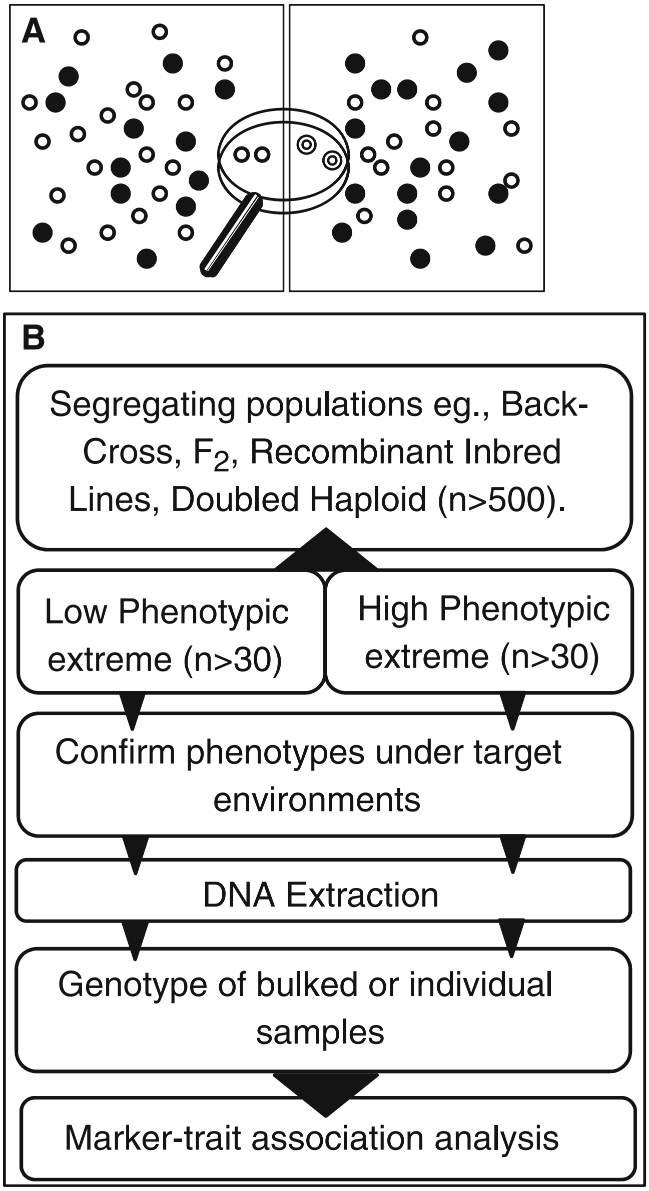

A
B
Segregating populations eg., Back-Cross, F2, Recombinant Inbred Lines, Doubled Haploid (n>500).
Low Phenotypic extreme (n>30)
High Phenotypic extreme (n>30)
Confirm phenotypes under target environments
DNA Extraction
Genotype of bulked or individual samples
Marker-trait association analysis

Figure 4. (**A**) SNP discovery scheme (magnifying glass)Illustrating common risk of ascertainment bias through limitation of sample numbers causing selection of non-representative or uninformative SNPs, and SNPs that cannot distinguish the two populations (Garvin *et al.* 2010). (**B**) Common strategy for marker–trait association analyses. Bioinformatics analyses compare sequence data and identify phenotype association. Marker Assisted Selection is dependent on efficient and effective phenotypic evaluation programmes (Xu *et al.* 2008).

such as rice (Yamamoto *et al.* 2010; Arai-Kichise *et al.* 2011; Gopala Krishnan, *et al.* 2011), wheat (Akhunov *et al.* 2009), maize (Yan *et al.* 2010), soybean (Kim *et al.* 2010) and grapes (Myles *et al.* 2010). Whole genome re-sequencing has been utilized in rice for identifying SNPs, taking advantage of the high-quality reference genome sequences of the japonica cultivar Nipponbare (IRGSP 2005). SNP resources based on differences between a few cultivars pose limitations in its utility as only a subset of them may be applicable to other combinations of genotypes. Therefore, emphasis has been placed on enlarging the SNP resources through re-sequencing of diverse *Oryza* accessions (Tung *et al.* 2010). Through whole genome re-sequencing using sequencing by synthesis, large numbers of SNPs have been discovered in japonica cultivars by comparison of Nipponbare with japonica cultivars Koshihikari (Yamamoto *et al.* 2010) and Omachi (Arai-Kichise *et al.* 2011). More recently, whole genome re-sequencing of six elite indica rice parental inbreds (three CMS and three restorer lines) have been carried out resulting in discovery of 2,819,086 non-redundant DNA polymorphisms including 2,495,052 SNPs, 160,478 insertions and 163,556 deletions across the rice genome, providing an average of 6.8 SNPs/kb across the genome, which is significantly higher than the previously reported SNP rates in rice (Gopala Krishnan *et al.* 2011).

Whole genome re-sequencing of the A genome wild relatives from Australia, *O. rufipogon* and *O. meridionalis*, has aided discovery of a large number of SNPs with a mean SNP rate of 6.71 and 6.33 SNPs/kb across the rice genome. A comparison of the SNP distribution across the rice genome between these wild relatives based on whole genome re-sequencing reveals that the elite cultivars have lost huge variability due to selection and crop improvement (Gopala Krishnan *et al.* unpublished). Relative to the Nipponbare reference sequence, the mean number of non-synonymous SNPs (nsSNPs) discovered per kb in 30,294 genes across the genome in the wild rice accessions is more than twice (0.89 and 0.87 nsSNPs/kb in *O. rufipogon* and *O. meridionalis*, respectively) compared to cultivated rice (0.30 nsSNPs/kb).

High levels of homology and gene synteny based on whole genome sequencing allows knowledge of gene function to be applied across species by comparison to high-quality reference genome sequences. Whole genome re-sequencing has been employed to study the patterns of evolution during the process of domestication and crop improvement in rice (He *et al.* 2011), maize (Gore *et al.* 2009) and soybean (Kim *et al.* 2010); discovering novel alleles for *crtRB1* gene in maize (Buckler *et al.* 2010); for genome-wide association studies of 14 different traits in rice and southern leaf blight of maize ; and for studying the genome-wide variations between parental lines of hybrids in rice (Krishnan *et al.* 2011) and maize (Lai *et al.* 2010).

3.4 *Gene-enriched genome sequencing*

Sequencing gene-rich fractions rather than whole genomes is much more efficient for the discovery of polymorphisms within genes. Polymorphisms detected within and around genes (as opposed to outside genes) are much more likely to be phenotypically relevant (i.e. to influence traits of interest) or to remain in linkage disequilibrium with causal mutations in the same gene through generations and across populations. The technique of genome enrichment can be used to define allelic differences between genotypes across the whole genome. Probes that are designed to hybridize with each of the genes in the genome can be used to isolate a genomic fraction enriched for complementary gene sequences for sequencing. The development of this technique has been driven particularly by the desire to discover rare sequence variants from the exome of the human genome (Frazer *et al.* 2009). Extremely promising results were achieved using probe sequences that are synthesized on microarray slides – the microarray probes capture the genomic DNA, which is then eluted from the microarray and sequenced (Albert *et al.* 2007; Okou *et al.* 2007). Using this approach, Hodges *et al.* (2007) designed a number of arrays to capture the entire human exome. A technical improvement on array hybridization is to carry out the hybridization in solution with free oligonucleotides using the microarray as a convenient platform only for oligonucleotide synthesis (Gnirke *et al.* 2009). Enrichment would appear to be a highly desirable approach for the discovery of large numbers of polymorphisms in plants although few reports using plant genomes have been published. A two-stage capture protocol was tested on maize targeting a chromosomal region and 43 dispersed maize genes (Fu *et al.* 2010). The first stage involved subtracting repeats, and in the second stage the targets were captured for sequencing. The solution hybridization-based approach has been used to enrich the sugarcane genome using probes mainly designed from the Sorghum genome sequence (Bundock *et al.* 2012) with hundreds of thousands of SNPs discovered within the polyploid sugarcane genome for 13000–16000 target genes.

3.5 *Organelle sequencing*

Historically, organellar sequences have been an important source of plant molecular markers. Early approaches to whole plastome sequencing involved either prior isolation of organellar DNA or PCR amplification. These approaches have been used in conjunction with second-generation sequencing to generate plastid genome sequences, producing large numbers of SNP and SSR markers for phylogenetic, barcoding and intraspecific studies (e.g. Cronn *et al.* 2008; Atherton *et al.* 2010; Doorduin *et al.* 2011; Zhang *et al.* 2011). Chloroplast (cp) isolation was reportedly ineffective as sequence reads from cp extracts contained over 80% non-cp DNA, whereas sequenced PCR amplicons had up to 24% non-cp DNA but required the use of universal primers.

Organelle sequencing is a routine outcome of sequencing DNA from plants because of the high number of copies of these genomes in the cell relative to the nuclear genome (figure 5). The chloroplast or mitochondrial sequences in the sample will usually greatly outnumber the nuclear sequences and, by assessing relative coverage, are easily distinguished from any organellar-like sequences resulting from genetic transfer to the nuclear genome. Sequencing of a total DNA sample can be used to readily determine the sequence of the chloroplast without prior purification or amplification of plastid DNA (Nock *et al.* 2011; Straub *et al.* 2011). This approach has been applied in phylogenetic analysis of plant species (Waters *et al.* 2012).

In comparison to animals, the mitochondrial genome of plants is characterized by structural complexity and highly variable size, ranging from 200 to 2400 kb (Ward *et al.* 1981). Second-generation sequencing of plant mitochondrial genomes has been used to identify markers involved in important crop breeding traits such as cytoplasmic male sterility (Fuji *et al.* 2010). It also reveals the prevalence of recombination and DNA transfer to the mitochondria from the chloroplast and nucleus and by horizontal gene transfer (e.g. Rodríguez-Moreno et al. 2011; Alverson *et al.* 2011). Large-scale sequencing of plant organelles will accelerate the discovery of markers for use in plant identification, population and phylogenetic studies; and improve understanding of DNA exchange among the organellar and nuclear genomes within the plant.

3.6 *Epigenetic markers*

Epigenomics refers to the large-scale study of epigenetic features on the genome, which include covalent modifications of histone tails (acetylation, methylation, phosphorylation and ubiquitination), DNA methylation and small RNA machinery (Rival *et al.* 2010). Bisulphite sequencing

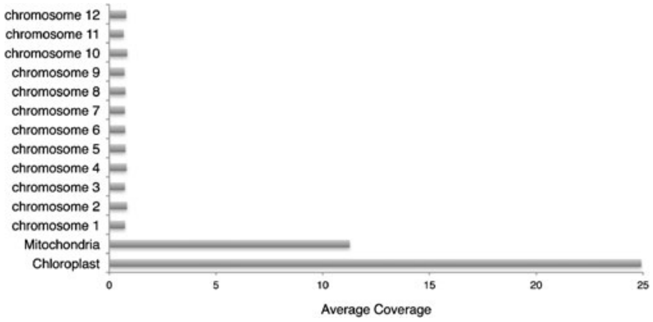


Figure 5. Relative coverage of the 12 rice chromosomes, mitochondrial and chloroplast genomes. From reference assembly of resequenced *Oryza sativa japonica* to the reference genome sequence (single lane of 35 bp paired-end reads; Illumina GAII).

involves the pre-treatment of DNA with sodium bisulphite, which causes deamination of cytosine to uracil, unless this reaction is blocked by methylation at the 5-carbon position. Third-generation sequencing technologies are capable of detecting sites of methylation directly. The delineation of regional DNA methylation patterns, and broader DNA methylation profiles, has important implications for understanding why certain regions of the genome can be expressed in specific developmental contexts and how epigenetic changes might enable aberrant expression patterns and disease (Laird 2010). Epigenetic phenomena have economic relevance in the case of somaclonal variation: a genetic and phenotypic variation among clonally propagated plants from a single donor genotype (Rival *et al.* 2010). Identification of heritable, epigenetic markers linked to important traits have potential in marker-assisted breeding.

3.7 *Genetic markers from high-throughput sequencing*

Genetic markers have played a major role in our understanding of heritable traits. As the cost of genome sequencing continues to decrease, it will become routine to re-sequence the genome of individual plants in place of the targeted genotyping with current SNP platforms (Imelfort *et al.* 2009b). MAS allows the breeder to achieve early selection of a trait in a breeding program. However, the vast majority of SNP are not readily associated with agronomic traits (Edwards and Batley 2010). While a few simple traits have been well characterized at the plant genome level, there are many other traits that are poorly understood. This is particularly true for complex traits that are controlled by interacting gene networks. The functions of many of the genes identified by genome sequencing remain unknown and the genetic control of the majority of agronomic traits is yet to be determined (Edwards and Batley 2010). Improvement of complex polygenic traits may be possible in the future by combining the application of newer technologies based on high-throughput genotyping associated with newer marker systems (e.g. SNP), and new selection strategies such as AB-QTL, Mapping-As-You-Go, Marker-Assisted Recurrent Selection and Genome-Wide Selection (Gupta *et al.* 2010).

Genetic mapping places molecular genetic markers in linkage groups based on their co-segregation in a population. The development of these markers also allows the integration of genetic and physical maps. The use of common molecular genetic markers across related species permits the comparison of linkage maps. Knowledge of the gene underlying a particular trait enables the transfer of the trait between cultivars and even species using genetic modification. Furthermore, the integration of molecular marker data with genomics, proteomics and phenomics data allows researchers to link sequenced genome data with observed traits (Edwards and Batley 2010). Advanced genome sequencing technologies generate large quantities of molecular marker genotyping data, which favours Association Studies over traditional QTL mapping (Edwards and Batley 2010).

4. Developments in plant molecular genetics driven by advances in sequencing technology

Molecular marker technology in plants is no longer limited by the capacity to discovery polymorphisms. Whole genome sequencing has been shown to now be a viable strategy for discovery of gene variants in the complex genomes of distant wild relatives of crops (Malory *et al.* 2011). Tools for polymorphism analysis are probably again the limiting factor. Genotyping by sequencing is an option that will possibly overcome this limitation. Ultimately all genotyping will be based upon determining the complete DNA sequence of every sample. Technological developments continue to make this a more realistic option. Species with simple genomes or for which a high-quality reference genome exists are most amenable to this approach using existing technology. These tools will deliver large numbers of genetic markers for variety identification and marker-assisted selection (Pattemore *et al.* 2010; Sexton 2010a and 2010b). Sequencing of plant populations will increasingly allow analysis of population and evolutionary genetics at the whole genome level (Fitzgerald *et al.* 2011; Shapter *et al.* 2012). These developments will deliver new insights into plant biology and support conservation of biodiversity, and plant improvement to deliver food and energy security (Henry 2010).

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